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Odpovídající role chondroitin sulfátu proteoglykanu, agrekanu, v denzní extracelulární matrix perineuronálních sítí a gliové jizvy

Matching the role of chondroitin sulphate proteoglycan, aggrecan, in dense extracellular matrix of perineuronal nets and glial scar

Bachelor´s thesis

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Prehlásenie

Prehlasujem, že som záverečnú prácu spracovala samostatne a že som uviedla všetky použité informačné zdroje a literatúru. Táto práca, ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

V Prahe, dňa 3.2.2020

Lenka Gmiterková

Poďakovanie

V prvom rade by som sa chcela poďakovať svojmu školiteľovi RNDr. Jiřímu Růžičkovi, Ph.D. za cenné rady a odbornú pomoc. Takisto ďakujem svojej rodine, ktorá mi bola počas písania tejto bakalárskej práce významnou oporou.

Abstract:

Traumatic brain and spinal cord injuries are worldwide medical problems. Disruption of the tissue leads to the changes in the cellular and extracellular matrix composition. This newly formed scar is not permissive for the axonal regrowth. Its function in prohibiting neuronal plasticity is similar to the perineuronal nets present in undamaged brain. One of the key components of both perineuronal nets and scar is proteoglycan aggrecan. In this thesis I focused on the function of aggrecan in central nervous system, mechanism of its growth inhibitory feature and research in the field of traumatic brain or spine cord injury treatment. It is important topic, since currently there are not any approved human therapies to recover axonal growth at the site of formed scar.

Keywords: extracellular matrix, perineuronal nets, aggrecan, traumatic brain injury, spinal cord injury

Abstrakt:

Traumatické poranenia mozgu a miechy sú celosvetovým zdravotníckym problémom. V poškodenom tkanive dochádza k zmenám na úrovni buniek aj medzibunkovej hmoty, kvôli čomu vzniká jazva. Zmena, ku ktorej dochádza v tkanive na mieste jazvy znemožňuje obnovu poškodených neurónov. Podobne ako jazva, ktorá bráni v nervovej plastickosti po poranení, funguje v nepoškodenom mozgu perineuronálna sieť. Jedným z kľúčových prvkov perineuronálnych sietí a jaziev v centrálnej nervovej sústave je proteoglykán agrekán. V tejto práci som sa zamerala na funkciu agrekánu v mozgu a mieche. Najmä na mechanizmus, ktorým bráni rastu neurónov a takisto na výskum v oblasti liečby týchto poranení. Táto téma je dôležitá, pretože momentálne neexistuje štandardná liečba, ktorá by obnovovala rast neurónov na mieste vytvorenej jazvy.

Kľúčové slová: medzibunková hmota, perineuronálne siete, agrekán, traumatické poranenie mozgu, traumatické poranenie miechy

List of Abbreviations

AAV – adeno-associated virus

ADAMTS – a disintegrin and metalloproteinase with thrombospondin motifs

AGC – aggrecan

ChABC – chondroitinase ABC

CNS – central nervous system

CRP – complement regulatory protein

CS – chondroitin sulphate

CSPG – chondroitin sulphate proteoglycan

ECM – extracellular matrix

EGF – epidermal growth factor

GABA – γ-aminobutyric acid

GAG – glycosaminoglycan

HA – hyaluronan

HAPLN – hyaluronan and proteoglycan link protein

HAS – hyaluronan synthase

LAR – leukocyte common antigen-related

MMP – matrix metalloproteinase

NG2 – nerve-glial antigen 2

NGF – nerve growth factor

Otx2 – orthodenticle homeobox 2

PNN – perineuronal net

PTPσ – protein tyrosine phosphatase σ

PV+ – parvalbumine positive

SCI – spinal cord injury

TBI – traumatic brain injury

WFA – Wisteria Floribunda Agglutinin

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1 Introduction

In my thesis I focused mainly on the function of aggrecan – central nervous system extracellular matrix component. I examined both the function in the physiological state and at the site of traumatic brain or spine cord injury. In the healthy brain chondroitin sulphate proteoglycans, including aggrecan, form perineuronal nets, rigid extracellular matrix structures enveloping certain neurons and synapses. Perineuronal nets regulate neuronal plasticity - growth of neurons and formation of new synapses. They play a role in signalling in synapses, memory formation and prohibition of neuronal growth.

Glial scar, which forms after central nervous system trauma has similar extracellular matrix composition like perineuronal nets. This scar is formed by glial cells, proteoglycans, link proteins and regulatory molecules bound to them. Changes in the cell and extracellular matrix composition might cause the impairment of the correct brain function. Chondroitin sulphate proteoglycans in the scar prohibit neuron outgrowth and therefore functional recovery of cut axons.

Based on the known facts I compared the role of aggrecan in perineuronal net and glial scar. In the perineuronal net it interlinks other components, thanks to that neurons surrounded by perineuronal net are stabilised and neuronal plasticity in the adult brain is reduced. Similarly, structure formed in the glial scar prohibits recovery of damaged neurons. I tried to explain, what is the mechanism of aggrecan inhibitory effect. Another important aspect of my thesis is the treatment of brain and spinal cord injuries. I researched, which modifications of aggrecan or extracellular matrix in general could help the recovery. With respect to the possible therapy, I have considered functional changes in various knockout mice strains and effects of some exogenous and endogenous enzymes, which cleaves brain extracellular matrix components and their impact on the neuronal plasticity.

This thesis aims to explain the complex role of aggrecan in brain extracellular matrix and emphasize its importance for the correct functioning. This knowledge could be further used in the future research of traumatic injury and neurodegenerative diseases treatment and memory and learning mechanism.

2 Perineuronal net structure and function

Perineuronal nets (PNNs) are specialized part of extracellular matrix (ECM) in central nervous system (CNS). Around 10-20% of adult brain is extracellular space filled with extracellular matrix (Cragg, 1979). There are different types of ECM in CNS; basement membrane, which is between the endothel and astrocytes, relatively loose matrix in parenchyma and condensed perineuronal nets around neurons (Lau *et al.*, 2013). Similar structure like PNN is perisynaptic matrix at the site of neuron connection. Even Ranvier nodes of myelinated axons can be enveloped by ECM structure called perinodal ECM (Fawcett, Oohashi and Pizzorusso, 2019). PNNs are not distributed evenly, only certain groups of neurons in the brain are enveloped in the PNN. The most prevalent are GABAergic (γ-aminobutyric acid) parvalbumine positive (PV+) interneurons. PV+ neurons are fast-spiking neurons signalling mostly to the neuronal soma. Another PNNs rich cell type are pyramidal cells, they are especially important in the CA2 region of hippocampus (Lensjø *et al.*, 2017). Cells which have perineuronal nets on their surface have enveloped neuron soma and dendrites. PNNs are localized in some areas of cortex, amygdala, hippocampus, cerebellum and spinal cord (Spijker and Kwok, 2017).

2.1 Perineuronal net composition

Perineuronal net is formed from many components, which occurrence, quantity and form can differ in various CNS regions. In general, they consist of hyaluronan, proteoglycans and link proteins. Hyaluronan is anionic structural backbone of perineuronal nets (Brockner *et al.*, 1993). Most of the proteoglycans in PNNs are lecticans namely aggrecan, neurocan, brevican and versican (Yamaguchi, 2000). They are attached to the hyaluronan by hyaluronan and proteoglycan link proteins (HAPLN) and between each other by tenascin-R (Carulli *et al.*, 2006; Morawski *et al.*, 2014) (Figure 1).

These components are synthetized by both neurons and glia. Hyaluronan and connective molecules are synthesized only by PNN ensheathed neurons. Production of neurocan is carried out by both the neurons and glia (Carulli *et al.*, 2006). Aggrecan is produced exclusively by neurons (Giamanco and Matthews, 2012). Versican and brevican are expressed only in glia, especially in astrocytes (Yamada, Watanabe and Yamaguchi, 1994).

Aggrecan, neurocan, brevican and versican are proteoglycans, which means, that they contain core protein and attached glycosaminoglycans (GAG) (Ruoslahti, 1988). More specifically they

belong to the lecticans, group of proteoglycans with homologous sequences, containing chondroitin sulphate as GAG bound in the central domain, N-terminal hyaluronic acid binding G1 domain and tenascin binding G3 domain (Ruoslahti, 1996).

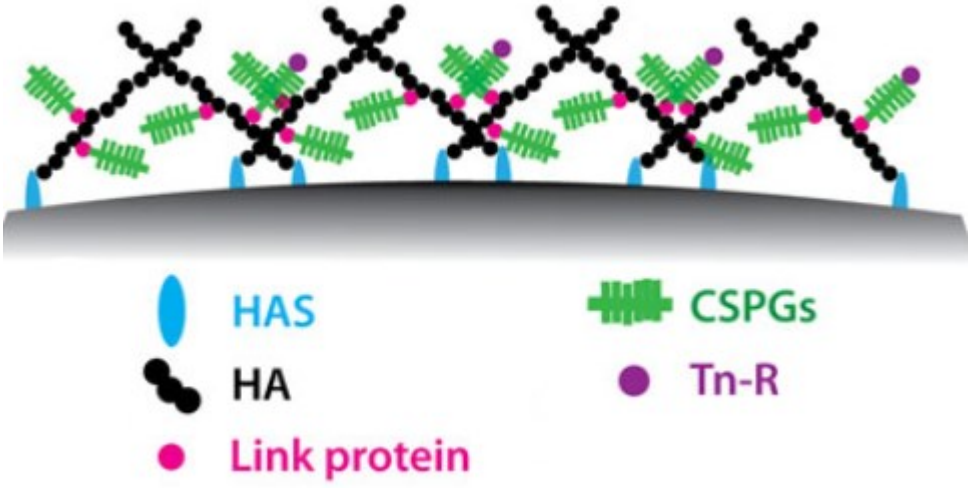

Figure 1 Schematic picture of perineuronal net bound to the neuron. Hyaluronan synthase (HAS, blue) is membrane bound enzyme producing hyaluronan (HA, black). Link protein (pink) binds chondroitine sulphate proteoglycans (CSPGs, green) – aggrecan, neurocan, brevican, versican - to the HA. Tenascin-R (Tn-R, purple) binds CSPGs. Modified from (Wang and Fawcett, 2012)

2.1.1 Hyaluronan

Hyaluronan (hyaluronic acid, HA) is expressed in the whole body, in CNS it serves as scaffold for perineuronal net, it is one of the necessary components for PNN formation (Kwok, Carulli and Fawcett, 2010). HA takes up the largest volume of PNN space and all other components are bound to it. Hyaluronan is glycosaminoglycan composed of repeating glucuronic acid and acetylgalactosamine subunit (Meyer, 1951). It is produced by membrane bound hyaluronan synthase (HAS) and secreted into extracellular space. There are three HAS isoforms - HAS1, HAS2 and HAS3. Expression of isoforms differs in various brain parts (Carulli *et al.*, 2006). HAS1 has role in the inflammation onset. *Has1* knockout is viable (Mack *et al.*, 2012). On the other hand, *Has2* knockout is lethal during embryogenesis (Camenisch *et al.*, 2000), HAS2 enzyme plays crucial role in ECM formation and therefore organism cannot live without it (Huang *et al.*, 2016). *Has3* knockouts are also viable (Mack *et al.*, 2012). HAS3 functions mainly as HA synthesis regulator (Arranz *et al.*, 2014). HAS3 is making a hyaluronan complexes of lower molecular mass than other two (Itano *et al.*, 1999).

2.1.2 Link proteins and tenascins

Both link proteins and tenascins are important in the PNN formation and reinforcement. HAPLNs are link proteins binding hyaluronan to the G1 domain of lectican. There are four

different types - HAPLN1 to HAPLN4. Their genes are paired with lecticans on chromosomes (Spicer, Joo and Bowling, 2003). One HAPLN type can bind more than one type of lectican (Shi *et al.*, 2004). HAPLNs are important for the perineuronal net formation. Brain of knockout mice without them remains in the PNN plasticity state (Carulli *et al.*, 2010). Another type of connecting molecules, tenascins, contain four different glycoproteins, but only tenascin-R and tenascin-C are present in brain ECM (Bourdon *et al.*, 1983; Wolff, Rathjen and Chiquet-Ehrismann, 1991). They are bound to the G3 domain of lecticans (Aspberg *et al.*, 1997) as well as cell surface molecules (Katoh *et al.*, 2013). Tenascin-R knockout is viable, but there are changes in PNN morphology, indicating importance of tenascin-R in hyaluronan and proteoglycan connection (Morawski *et al.*, 2014).

2.1.3 Neurocan

Neurocan is chondroitin sulphate proteoglycan (CSPG). It is specific for CNS and also one of the main proteoglycans of perineuronal nets. Neurocan has N-terminal and C-terminal globular domains binding HAPLN and tenascin-R. Neurocan is expressed already in embryonic state and in short time after birth its concentration starts to decrease (Engel *et al.*, 1996). Levels of neurocan are small, because it is continuously cleaved by matrix metalloproteinase (MMP) enzyme. In the adult brain only cleaved fragments can be found (Rauch *et al.*, 1991). Later in life it can be again upregulated at the site of glial scar, formed after traumatic brain injury or stroke (Carmichael *et al.*, 2005). Higher levels of neurocan were observed also in the epilepsy (Heck *et al.*, 2004). Neurocan knockout mice is viable and does not show apparent morphological changes. Its function is probably subtle and can be substituted by other lecticans (Zhou *et al.*, 2001). Multiple knockouts eliminating also other lecticans, such as brevican, help to explain its function since they cannot compensate for neurocan deficiency anymore (Gottschling *et al.*, 2019).

2.1.4 Brevican

Brevican is another brain-specific chondroitin sulphate proteoglycan. Brevican is expressed only by astrocytes, not by neurons. Structure is similar to neurocan and aggrecan, it contains G1 domain for hyaluronan binding, central GAG binding domain and lectin binding G3 domain (Yamada, Watanabe and Yamaguchi, 1994). In addition to excreted form, brevican can exist in membrane bound form using GPI-anchor. Expression of brevican increases in the adulthood (Seidenbecher *et al.*, 1995). Brevican levels are also temporarily increased after traumatic brain injury (Jones, Margolis and Tuszynski, 2003). Brevican knockout mice are viable and without

morphological changes, but with functional changes in the memory formation process. Neurocan is upregulated after brevican deletion, so it might compensate its function (Brakebusch *et al.*, 2002). Double brevican and neurocan knockouts show their role in axon growth guidance and restriction. Neuronal growth in the brevican and neurocan deficient mice was recovered after injury because of decreased inhibitory effect (Quaglia *et al.*, 2008). Quadruple knockout of tenascin-R, tenascin-C, neurocan and brevican decreased the amount of PNNs in CA2 hippocampal region, which subsequently decreased abundance of inhibitory synapses and induced neuronal activity (Gottschling *et al.*, 2019).

2.1.5 Versican

Versican is CSPG from lectican family – it has domain binding to the hyaluronan through HAPLN and tenascin binding domain. It can be found in various tissues, brain being one of them. There are several isoforms of versican produced by alternative splicing of exons called V0, V1, V3 and V4. They differ in the number of bound GAGs (Dours-zimmermann and Zimmermann, 1994). Isoform V3 does not contain any bound GAGs and therefore it is not proteoglycan (Zako *et al.*, 1995). Brain versican is produced only by glia, not by neurons (Asher *et al.*, 2002). Versican can be detected already in the embryonic stage, then its quantity lowers postnataly and again rises in the adulthood (Milev *et al.*, 1998). Versican is inhibitor of axonal growth as well (Schmalfeldt *et al.*, 2000). Its knockouts are not viable, but partial knockout of G1 subdomain caused change in the structure of ECM and lowered cell migration during ontogenesis (Hatano *et al.*, 2012).

2.1.6 Phosphacan

Phosphacan is chondroitin sulphate proteoglycan present in perineuronal nets, it does not belong to the lecticans. Phospahcan is secreted, extracellular splicing variant of transmembrane receptor-type protein tyrosine phosphatase, lacking transmembrane and intracellular domain (Maurel *et al.*, 1994). It is present around the synapse of some neurons (Hayashi, Oohira and Miyata, 2005). And also around astrocytes at the site of injury. At the injury site, it prohibits the neuron outgrowth (Snyder *et al.*, 1996). Its levels are elevated after CNS trauma. Secreted phosphacan form serves during new synaptogenesis after injury (Harris, Reeves and Phillips, 2012). Even though phosphacan is not one of the lecticans it binds tenascin and also neural cell adhesion molecules, because of that phosphacan plays an important role in regulation of neuron and glia adhesion (Grumet *et al.*, 1994; Milev *et al.*, 1994). Perineuronal nets in phosphacan

knockout mice had decreased cell-adhesion abilities. After addition of soluble phosphacan PNNs regained their wild type morphology (Eill *et al.*, 2019).

2.1.7 Aggrecan

In addition to perineuronal nets, aggrecan (AGC) is present in articular cartilage and is also important in growth plate formation in skeleton. Encoded by *ACAN* gene, aggrecan is composed of three globular domains and central domain. First is N-terminal globular domain (G1 domain), which consists of three cystein loops. G1 binds hyaluronan via link proteins. Aggrecan contains also G2 domain, which is not present in versican, brevican or neurocan. Its function is unknown, even though it is highly homological to G1 domain, it does not bind hyaluronan (Watanabe *et al.*, 1997). Downstream of G2 domain, in central domain, are keratan sulphate binding sites (Doegesq, Sasakill and Kimurajj, 1991), which are not occupied in PNN aggrecan, but cartilage aggrecan instead contains keratan sulphate. Main part of central domain has around 100 binding sites for chondroitin sulphate (CS) (Hardingham, 1981). C-terminal globular domain (G3 domain) contains one or two EGF (epidermal growth factor) repeats, according to present splice variant, C-type lectin domain and CRP-like (complement regulatory protein) domain. It can bind various carbohydrates and GAGs, tenascin-R binds there contributing to the condensation of PNNs matrix (Aspberg *et al.*, 1997) (Figure 2).

Figure 2 Aggrecan structure with three globular domains (G1, G2, G3) and chondroitin sulfate chains (green) and keratane sulfate chains (pink). Modified from (Yamaguchi, 2000)

While protein and GAG composition of aggrecan is stable, sulfation pattern on the chondroitin sulphate chains changes dynamically, which affects aggrecan stability. In case of more 6-sulfation than average in adult brain, it is more prone to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) degradation and thus destabilizes perineuronal nets. This is normal process in the developing brain which undergoes plasticity (Miyata and Kitagawa, 2016). Because of that, aggrecan can be first detected in increased amount only at the end of plasticity period (Hockfield *et al.*, 1990). In developed brain after end of plasticity period sulfation pattern has more 4-sulfation than 6-sulfation. This form of aggrecan is more firm and helps to stabilize PNNs and synapses (Miyata and Kitagawa, 2016).

In developing brain, aggrecan regulates differentiation of glial cells to the astrocytes by lowering the number of their precursors (Domowicz *et al.*, 2008). Also, aggrecan level is upregulated in the absence of glia (Giamanco and Matthews, 2012). This shows their reciprocal regulation in creating suitable environment.

There are diverging opinions on the necessity of aggrecan presence during PNNs formation. Some studies show, that aggrecan is essential component, for example *in vivo* study by Rowlands found out, that in absence of AGC because of deletion of *ACAN* gene, perineuronal nets and surrounded neurons remained in the juvenile plasticity state (Rowlands *et al.*, 2018). On the other hand *in vitro* and *ex vivo* study of PNN components distribution, in the aggrecan deficient cmd knockout mice and chondroitinase ABC (ChABC) treated slices, detected net-like structure of hyaluronan and link proteins even in the absence of aggrecan (Giamanco, Morawski and Matthews, 2010). Explanation might be either that PNNs can form without aggrecan, but they would be functionally impaired (Rowlands *et al.*, 2018) or that there are aggrecan free PNNs with unknown function. Structure, which has other PNN components, but no aggrecan, was described in visualization experiment by Ueno (Ueno *et al.*, 2018).

Perineuronal nets can be visualized by molecules binding to the GAGs. Most commonly used for the whole PNNs visualization is Wisteria Floribunda Agglutinin (WFA) fluorescent staining, which binds to the N-acetylgalactosamine on the GAG of chondroitin sulphate proteoglycans (Härtig, Brauer and Bruckner, 1992). WFA is not present after chondroitinase ABC treatment or in the aggrecan knock-out, implying that it binds to the aggrecan (Giamanco, Morawski and Matthews, 2010). Alternatively, immunolabeling with antibodies can be used. They bind to the exact molecule or even exact isoform and as such they are not optimal for the whole PNN visualization. But they are important in uncovering site specific differences of brain extracellular matrix. Commonly used aggrecan antibodies are Cat-301 and Cat-315, binding protein core, Cat-316 binding GAG epitope and AB1031 binding to the protein core as well (Matthews *et al.*, 2002).

Cat-301, Cat-315 and Cat-316 antibodies bind aggrecan, but each of them different glycoform – same protein with different glycosylation pattern. In addition to that, there are cells expressing aggrecan mRNA, which are not stained by any of those three antibodies. Various glycoforms of AGC can be found in different brain regions. This may implicate, that each glycoform has different properties and different function in CNS. It may play a role in the physiological differentiation of brain and spinal cord (Matthews *et al.*, 2002). Additionally, not

all sites of aggrecan expression are WFA positive. Reason behind that might be, that even WFA is not broad aggrecan marker and probably localizes only certain glycosylation form. This form is present site-specifically in some brain parts. Other types of perineuronal nets, which are not stained by WFA, can contain different aggrecan form and even have changed ratio of other components (Yamada and Jinno, 2017). Appearance of WFA staining at the end of plasticity period and its reduction when 6-sulfation is increased indicates, that it stains some plasticity prohibiting type of aggrecan (Miyata and Kitagawa, 2016). Also there are WFA positive nets, which does not bind any other known aggrecan antibody. This could be explained in different ways. One is that we do not have the exact antibody which binds to this type of aggrecan. Another opinion is, that WFA does not stain aggrecan, but some currently unidentified molecule in PNNs (Ueno *et al.*, 2018). In case that WFA does not bind to the aggrecan, WFA stained PNN without other AGC antibody might be a type of aggrecan free perineuronal net.

2.2 Function of perineuronal nets and role of aggrecan

Perineuronal net main functions are protection against oxidative stress, stabilization of synapses and plasticity regulation. All those functions are part of PNNs neuroprotection. When the neuroprotective function fails various diseases can form. Its function can also diminish with the increased age.

Hyaluronan and lecticans have anionic structure, thanks to the saccharides. They give the whole PNN negative charge. This negative charge helps to protect against oxidative stress, anions capture oxido-reductive agents before they could get into the cell (Morawski *et al.*, 2004). Another function, which is partly related to the anionic nature is synapse stabilization, there are two types, hydrodynamic and mechanical stabilization. Hydrodynamic stabilization is based on capability of PNN to store cations in the synapses, they are bound to the GAGs by non-covalent bond. This way instead of diffusion of ions secreted during action potential, they stay in the synapse and participate in the extracellular space ion equilibrium. Afterwards, they can be used to restore cell equilibrium (Morawski *et al.*, 2015). Synapses are also stabilized mechanically by perineuronal nets, which in contrast with the rest of brain ECM are relatively rigid structure. They prohibit the formation of new synapses, because they envelop neurons at the place, where new synapse would potentially form (Hockfield *et al.*, 1990).

Yet another attribute of perineuronal nets is, that they bind various active molecules such as growth factors, cytokines or inhibitors of synaptic formation. One such inhibitor is semaphorine

3A, which binds to the chondroitin sulphate of lecticans and because of that reduces neuronal plasticity (Vo *et al.*, 2013). Orthodenticle homeobox 2 (Otx2) is transcription factor, it binds to the CS of proteoglycans as well. Otx2 binding increases, when sulfation changes in favour of 4-sulfation, which is normally present in adult brain, instead of 6-sulfation. This increased binding enables accumulation of Otx2 in PV+ neuron surroundings and therefore more of it is absorbed (Miyata *et al.*, 2012). Changes in cell expression caused by Otx2 help maturation of PV+ interneurons and are engaged in closure of plasticity period (Bernard and Prochiantz, 2016).

2.2.1 Role of aggrecan in perineuronal nets

Aggrecan plays an important role in oxidative stress protection and hydrodynamic synapse stabilization. It is PNNs lectican, which binds the highest amount of GAGs. While aggrecan has up to 100 GAG binding sites, neurocan has only one to three and brevican can be present even in GAG-free form. Because of that aggrecan together with hyaluronan are mainly responsible for PNN negative charge (Hardingham, 1981; Yamaguchi, 2000). Mechanical stabilization of synapses is related to the AGC as well. Even though PNN can form also in the absence of aggrecan, its presence is necessary to stabilize them and prohibit new neuronal growth (Rowlands *et al.*, 2018).

2.2.2 Neuronal plasticity and critical period

Levels of lecticans change during development in different patterns, their connection to the functional changes is not yet precisely determined. Need of the sensory stimulus during critical period for the formation of functioning neuronal network was first described in the cat with impaired sight. This cat did not obtain any sensory stimulus from the eyes, no active synapses were formed in the visual cortex and at the end of critical period PNNs did not form properly (Guimaraes, Zaremba and Hockfield, 1990). When such cat was exposed to the light, eyesight did not repair. After digestion of defective PNNs by chondroitinase ABC or hyalurodinase enzyme plasticity was restored and neuronal network with accompanying perineuronal net formed properly (Pizzorusso, 2002).

Critical period (CP) is time period during ontogenesis, when neurons in brain must obtain signals from sensory organs in order to form sustainable connections. From many synapses formed in the early stage of ontogenesis only those which are sending and receiving signals are preserved in adulthood. For their preservation, they need to be stabilized by PNNs (Ye and Miao, 2013) (Figure 3).

Plasticity can be reactivated in already formed nets experimentally by chondroitinase ABC, which cleaves GAGs including hyaluronan and chondroitin sulphates into disaccharides or hyalurodinase cleaving hyaluronan. Endogenous equivalent of hyalurodinase and chondroitinase are ADAMTS and matrix metalloproteinases (MMPs). ADAMTS are more potent in cleaving aggrecan and therefore dissolving perineuronal nets, than MMPs (Durigova *et al.*, 2011). Expression of both ADAMTS and MMPs is higher during development, when synapses are created and coincidents with the changes in plasticity (Wen *et al.*, 2018).

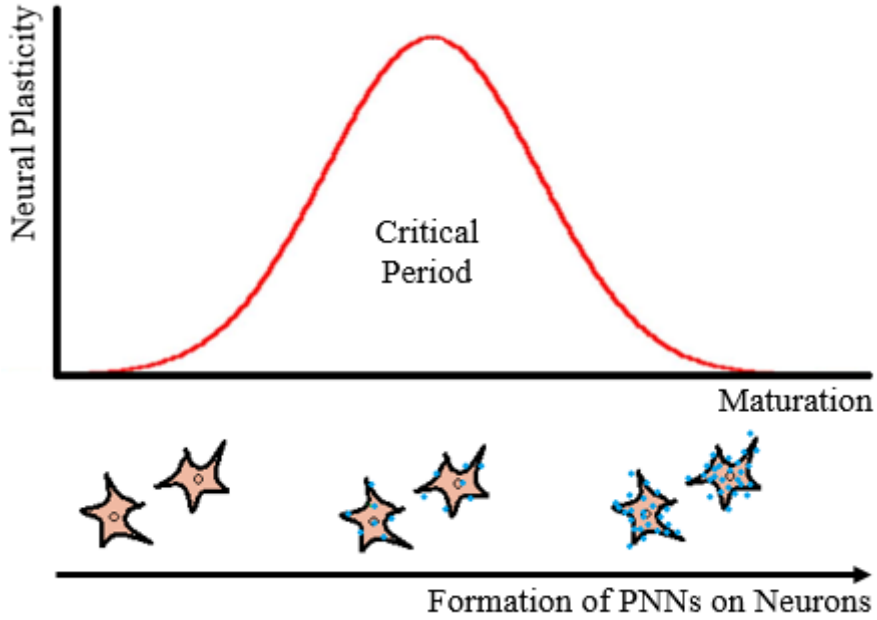

Figure 3 Graph of critical period showing, how neural plasticity increases at the onset of critical period together with increased PNN formation. At the end of CP perineuronal nets are fully formed and plasticity is prohibited.

2.2.3 Role of aggrecan in neuronal plasticity

Aggrecan has important role in prohibition of neuronal plasticity after critical period, without AGC they stay in the state of permanent plasticity (Rowlands *et al.*, 2018). At the end of CP aggrecan levels rise, interconnect the PNNs around synapses and stabilizes them. Other components of PNNs are already being produced from the earlier stage of ontogenesis in high levels since they are important in the synapse formation during plastic state (Ye and Miao, 2013). Maturation of neurons and both onset and closure of critical period are regulated by Otx2, which binds to the CS chain of glycoproteins. At least some lecticans with CS chain are needed to initiate plasticity. Onset of ocular plasticity was impaired in the PNNs with low CS amount. With the progression of critical period more CSs are produced and more Otx2 is bound. Since aggrecan has biggest amount of bound chondroitin sulphate chains, Otx2 binding and

consecutive absorption by cells are significantly elevated together with elevated aggrecan levels and when it reaches certain amount it initiates closure of critical period (Hou *et al.*, 2017).

2.2.4 Perineuronal nets and memory

In addition to PNNs activity during critical period in the developing organism, they play key role in the memory formation throughout the life. Plasticity prohibition by perineuronal net is important factor in learning and memory. In different brain regions we can observe changes in PNNs abundance. Sensory cortices have high density of PNNs, which coincides with their low plasticity after end of critical period. Higher association areas have smaller PNNs density and therefore they are more plastic and permissive for the reinforcement or reduction of synapses (Hendry *et al.*, 1988). That is very important in formation of new associations and long term memory. For example, fear memories became impossible to erase after closure of plasticity period, in contrast with fear memories from early age, which could be forgotten. In study by Gogolla they dissolved perineuronal nets in amygdala by chondroitinase ABC. Plastic state was reintroduced and fear memories could have been again forgotten (Gogolla *et al.*, 2009).

2.2.5 CNS pathologies related to the PNNs

Neuroprotective effect of perineuronal nets or its lost can be observed during some diseases. One of them is Alzheimer disease in which PNNs inhibits Tau diffusion throughout the brain and its internalization into the cells. Alzheimer disease plaques are widely spread in the parts of brain without PNNs, but not that much in the PNN rich regions. Perineuronal net forms mechanical barrier for Tau protein (Suttkus *et al.*, 2016). With aging we can observe reduction of PNNs in brain, this causes that older people are more susceptible to the Alzheimer disease (Brewton *et al.*, 2016). Other diseases connected to the PNNs include schizophrenia, pathology with decreased amount of PNNs causing neurodevelopmental defects (Mauney *et al.*, 2013) and epilepsy, in which aggrecan-positive PNN levels are decreased after seizure and therefore synaptic reorganization of inhibitory synapse is upregulated (McRae *et al.*, 2012). Mechanical disruption of tissues after stroke, traumatic brain injury and spinal cord injury cause degradation of PNN at the vicinity of injury (Harris *et al.*, 2010; Karetko-Sysa, Skangiel-Kramska and Nowicka, 2011) and formation of scar with abundance of PNN components at the place of injury (Fitch *et al.*, 1999).

3 Spinal cord and brain injury

Traumatic brain injury (TBI) or spinal cord injury (SCI) are caused by penetration of CNS tissue by foreign object or blow, which disrupts joined neurons, glial cells and ECM. Worldwide there are around 69 million of traumatic brain injury cases a year (Dewan *et al.*, 2019). Mild cases can naturally recover, but others can cause long-lasting damage in CNS function or even death. Major issues of CNS injury are destruction of neurons and long axons. After demise of neurons, astrocytes are left in the scar, they change their expression and turn into reactive astrocytes. Production of ECM components is elevated. This process is called gliosis and forms glial scar. All of this can happen only after damage to the blood-brain barrier. Degradation of neuron is not enough to develop protective inflammatory reaction (Fitch and Silver, 1997). Macrophages and molecules foreign to brain must get there, activate astrocytes and cause inflammation. TBI and SCI in most cases progress to the secondary injury. Such a scar is wider than original wound, contains ECM and glial cells as a barrier on the periphery and can cause dangerous changes in blood flow and intracranial pressure (Klusman and Schwab, 1997). Stroke can have similar effect on the brain tissue like traumatic brain injury (Karetko-Sysa, Skangiel-Kramska and Nowicka, 2011).

3.1 Glial scar

Glial scar plays important role in repair of blood-brain barrier and isolation of wound from healthy tissue. Inflammation can activate healing process, but balance between regrow of axons and permanent scar formation is not known yet. Glial scar is formed by reactive astrocytes, macrophages, microglia, fibroblasts and oligodendrocyte precursor cells also called NG2 (nerve-glial antigen 2) glia. NG2 glia are important part of scar, they migrate to the site of injury and there they are source of NG2 CSPG for the extracellular matrix. ECM of glial scar contains mostly CSPGs, heparan sulphate proteoglycans, tenascins and molecules bound to them (Levine, 1994; Bradbury and Burnside, 2019). Proteoglycan gradient is increasing toward the scar centre. Glial scar does not prohibit axonal growth simply by mechanical barrier, chondroitin sulphate proteoglycans and other neuronal plasticity inhibitory molecules bound in the matrix play an important role. Level of CSPGs abundance in glial scar is increased after the arrival of microglia and macrophages and damage to the blood-brain barrier. Microglia and macrophages produce soluble growth inhibitors and inflammation agents at the site of injury (Fitch and Silver, 1997). Inflammatory processes, astrocyte activation and glial scar formation

are activated even in the absence of injury, if inflammation molecules are present, which indicates, that main factor are those molecules, not immune system cells (Fitch *et al.*, 1999). Formation of glial scar is necessary not only for protection of blood-brain barrier, but also afterwards during healing. When scar formation was prohibited, neurons could not grow since there was not scaffold for them (Anderson *et al.*, 2016). Both growth activators and inhibitors, such as proteoglycans, are increased at the injury site, but inhibitors more (McKeon *et al.*, 1991).

3.1.1 Axon regeneration

In the past there was a theory, that axons of brain neurons do not regenerate (Gros Clark, 1943). Shortly afterwards it was discovered, that they can regrow, but their growth is inhibited because of unsuitable environment (Liu and Chambers, 1958). In contrast, axons can grow in peripheral nervous system without major inhibition (Richardson, McGuinness and Aguayo, 1980). When axon reaches glial scar it forms dystrophic endbulb form (Ramón y Cajal, 1930). Even this dystrophic ending can start growing in the suitable environment. Actually, they have stable turnover of membranes and cytoskelet even when they do not elongate (Tom *et al.*, 2004). Today there are several approaches in restarting the axonal growth in CNS, which was inhibited by ECM components, such as stimulation by growth factors, modification of extracellular environment or disablement of inhibitors (Niekerk *et al.*, 2016).

3.1.2 Axonal growth inhibitors and activators

Some of the CNS inhibitory molecules are always present and are only upregulated after injury, others are newly expressed. One of the constantly present is myelin ensheating healthy as well as collapsing neurons. After injury, demyelination of axons can start (Mierzwa *et al.*, 2015) and remaining myelin from crushed axons prohibits elongation of new axons at the place where it is still present (Wang *et al.*, 2002). Another inhibitor is tenascin, its production is upregulated in reactive astrocytes (Apostolova, Irintchev and Schachner, 2006). One of the major inhibitors are chondroitin sulphate proteoglycans. In healthy brain only some parts have considerable amount of CSPGs, namely perineuronal nets. After injury, reactive astrocytes produce CSPGs, which work as axonal growth inhibitor, at any part of brain (Smith-Thomas *et al.*, 1994). CS chains of all CSPGs prohibits neuronal plasticity, while core proteins can have various properties (Lemons *et al.*, 2003). Also semaphorine 3A, very potent neuron chemorepellent, is produced by fibroblasts at the core of scar (Pasterkamp, Anderson and Verhaagen, 2001). Semaphorine is changed from the guidance molecule to the repulsive one after binding to the

proteoglycan (Kantor *et al.*, 2004). Ephrins, which under normal circumstances serve in axonal pathfinding and cell migration in nervous system development are upregulated after injury and maintain the axon repelling (Miranda *et al.*, 1999). Those and other such molecules bound to the CSPGs might play an important role in its inhibitory activity.

Formation of permanent glial scar is not caused only by presence of inhibitors but also by absence of growth activators. It is needed to both stimulate the regeneration and surpass the prohibition in order to fully heal the scar (Steinmetz *et al.*, 2005). One such growth promoting factor is laminin. In its absence axons cannot recover even when inhibitors are removed (Grimpe *et al.*, 2002). Laminin serves as scaffold for new axon. In CS gradient, growth cone might upregulate laminin receptors or downregulate CS receptors while it proceeds through changing enviroment (Snow and Letourneau, 1992). Rising of cAMP levels in neuron helps to stimulate its growth even in inhibitory environment (Qiu *et al.*, 2002).

3.1.3 Changes of CSPGs in glial scar and its surroundings

Glial scar at the site of injury functions as protection of blood-brain barrier, while decreased amount of CSPGs in the surroundings helps recovery. After TBI, composition of space around the injury changes for some time to the more permissive for regeneration. Transcription of inhibiting lecticans is lowered (Harris *et al.*, 2010). On the other hand, CSPG production in glial scar is already upregulated one day after injury and can stay this way up to few months. Neurocan and tenascin are upregulated right after injury, with peak after one week. Brevican and phosphacan reach the peak after one month (Tang, Davies and Davies, 2003). Aggrecan concentration in the glial scar is increased as well, two weeks after trauma (Yi *et al.*, 2012), but prior to that it is cleaved by MMPs and ADAMTS, which causes increased amount of aggrecan fragments and decreased aggrecan. Same cleavage process happens also in the vicinity of scar (Lemons *et al.*, 2001). Changes in versican expression are not clear (Tang, Davies and Davies, 2003; Yi *et al.*, 2012). There is also increased 4-sulfation of chondroitin sulphate proteoglycans at the site of injury, which further prohibits axon sprouting (Wang *et al.*, 2008). Studies of 6-sulfation are unclear, Properzi found that it was upregulated and had inhibitory effect as well (Properzi *et al.*, 2005), while Wang did not find any change in the growth restriction after 6-sulfation elimination (Wang *et al.*, 2008). Changes in CSPGs levels at the site of injury are same both in spinal cord injury and traumatic brain injury (Andrews *et al.*, 2012; Pearson *et al.*, 2019).

3.2 Role of aggrecan in the neuron growth

3.2.1 Stripe aggrecan model

In the study by Johnson *et al* they examined neurite growth on aggrecan. They used alternating laminin and aggrecan stripes with different AGC concentrations. In the beginning neurons were at the laminin stripe, number of neurites crossing aggrecan to the another laminin stripe was counted after 24 hours of incubation. In the sample with 10μg/ml of AGC neurites could grow at the aggrecan without visible problems, but at the concentration of 100μg/ml they are significantly hindered and in the majority they turned in the another direction (Johnson *et al.*, 2002) (Figure 4). This model best represents environment in the spine during ontogenesis, which permits correct pathfinding, but is not very good for glial scar modelling, where neurons do not turn but stop growing. Notable is also similar model of CSPG step gradient. It consists of adjoined stripes with increasing concentration of CSPGs bound to laminin layer. Growth cone starts at the lowest concentration and continues to grow to the higher instead of changing path at the border like it happens in aforementioned stripe model. This model shows us, that growing neuron is able to better adapt to the higher final concentration in the gradually increasing environment, than in case of bold change of surroundings composition. With increased CSPG concentration growth rate of axon decreases (Snow and Letourneau, 1992).

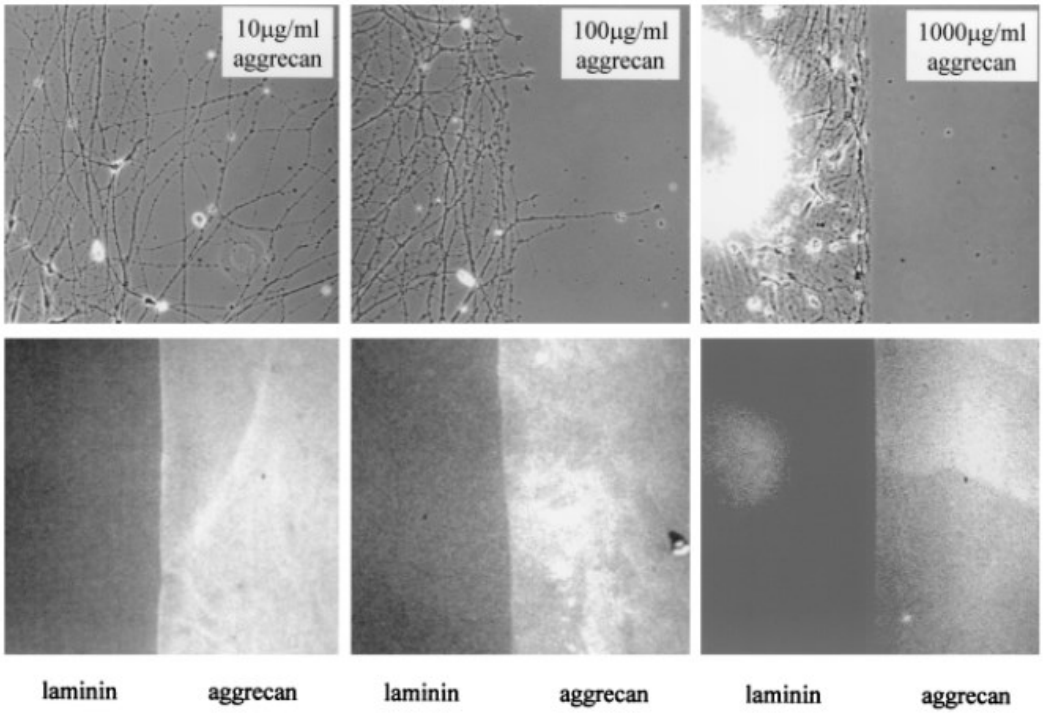

Figure 4 Cultures with alternating laminin and aggrecan stripes. Concentration of aggrecan is increased from the left to right and axonal growth into AGC stripe diminished. Instead they change the direction of growth alongside aggrecan/laminin range (Johnson *et al.*, 2002).

3.2.2 Spot gradient model

Glial scar has highest proteoglycan concentration in the centre and lower on the edge (Fitch *et al.*, 1999). Tom *et al* created spot gradient model in their *in vitro* study to better portray glial scar environment. It consists of laminin and aggrecan spots with increased AGC and lower laminin concentration in the outer circle. Spot gradient model is inversed version of glial scar, where concentration in centre is highest. Neurons were growing from the centre to the periphery, but only up to the certain point with too high aggrecan concentration. At this site, they formed dystrophic endbulbs similar to the ones at the injury site (Tom *et al.*, 2004) (Figure 5). This gradual model portrays glial scar much better, than stripe model.

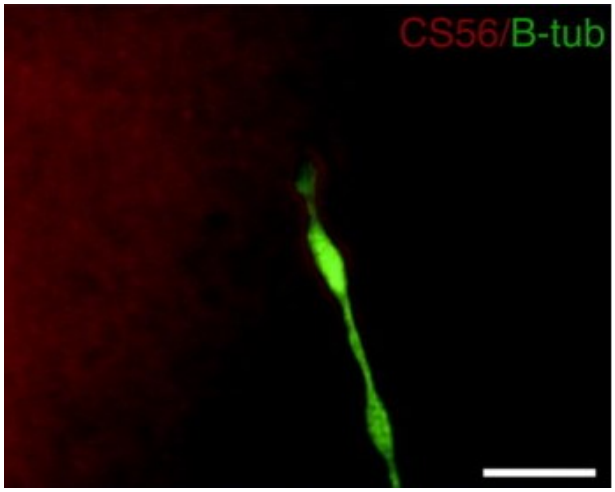

Figure 5 Spot gradient model of dystrophic endbulb (β-tubulin in green) of neuron growing in environment with increasing aggrecan (red) gradient. Scale bar 20 μm (Tom *et al.*, 2004).

3.2.3 Properties of glycosylated aggrecan and core protein

Concentration of aggrecan at the certain parts of glial scar is not the only important property. Form of the present aggrecan also plays a role. Aggrecan bound to hyaluronan and link protein naturally forms aggregate, called perineuronal nets. This aggregate prohibits the growth of neurons. Also aggrecan monomers, which are not bound together by linking molecules are prohibiting neuron growth in *in vitro* model implicating, that AGC itself has inhibitory properties (Chan, Roberts and Steeves, 2008).

In the *in vivo* study by Lemons *et al* they injected either aggrecan protein core or glycoprotein with CS into the rat spinal cord hemisection. Both of them proven to be growth prohibiting, showing that not just bound CS are inhibiting, but very aggrecan as well. Because of that, digestion of CS by ChABC might not be enough to overcome CSPGs inhibition in glial scar (Lemons *et al.*, 2003). In another *in vitro* study aggrecan core protein did not prohibit the growth of neuron, but observed morphological changes in axons were similar to the dystrophic

endbulbs formation. Number of filopodia was decreased and growth cone width increased (Beller *et al.*, 2013) (Figure 6). Also N-glycans, branched sugars bound to the G1 globular domain of aggrecan have inhibitory effect on the neurite growth, probably because they are binding neuronal cell adhesion molecules (Hering *et al.*, 2020). Aggrecan core protein has some inhibitory effect on the neuron outgrowth even though its extent is not clear. Undigested brain aggrecan contains CS chains, which are responsible for the majority of aggrecan inhibition in neurite growth (Bradbury *et al.*, 2002).

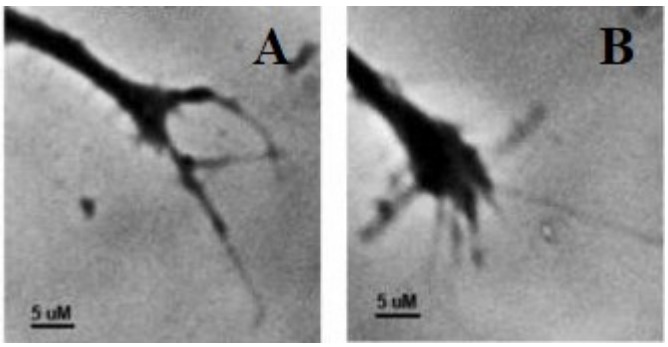

Figure 6 Stripe aggrecan assay without CS chains, (A) at the site without aggrecan, neurites grow normally, (B) when they reach AGC stripe, they become wider and number of filopodia decreases. Modified from (Beller *et al.*, 2013).

3.2.4 Protein tyrosine phosphatase σ as CS receptor

The protein tyrosine phosphatases contain various transmembrane cellular receptors; leukocyte common antigen-related (LAR) receptor, protein tyrosine phosphatase σ (PTPσ) and δ (PTPδ) receptor. Both LAR and PTPσ receptors are important in the axon growth regulation (Chagnon, Uetani and Tremblay, 2004). PTPσ is transmembrane protein which binds to the chondroitin sulphate of CSPGs with high affinity and functions as a cellular receptor. PTPσ knockout neurons were more likely to grow in the CSPG gradient than wild type neurons (Shen *et al.*, 2009). In the normal growing neurons PTPσ is evenly distributed on the cytoplasmatic membrane, but in the dystrophic cones it becomes concentrated and stabilize neurite ending on the CSPG substrate and therefore create dystrophic endbulb (Figure 7).

In the study by Lang *et al* they created peptide, which binds to the PTPσ and inhibits CS binding. In the *in vitro* gradient model, blockage by this peptide had same effect as ChABC treatment, growth of dystrophic cone was recovered. Too high concentration of peptide caused decreased neuronal adhesion and stop of the growth, showing the importance of CSPG as growth scaffold. This treatment used *in vivo* partially recovered functions, which were damaged during spinal cord injury. Only part of the cut axons was regenerated indicating that auxiliary help is needed for this type of treatment (Lang *et al.*, 2015). Similar approach is to use RNAi (ribonucleic acid

interference) in order to silent PTPσ gene and block its function. Virus with RNAi can be injected at the site of injury. It helps in the axon regeneration, but does not affect the glial scar formation (Zhou *et al.*, 2014). Sensitivity of PTPσ receptor depends also on the sulfation pattern of chondroitin sulphate GAGs. Chondroitin-4,6-disulphate is very potent at PTPσ binding and therefore promotes more efficient growth inhibition of the axon, than other sulfation patterns (Brown *et al.*, 2012).

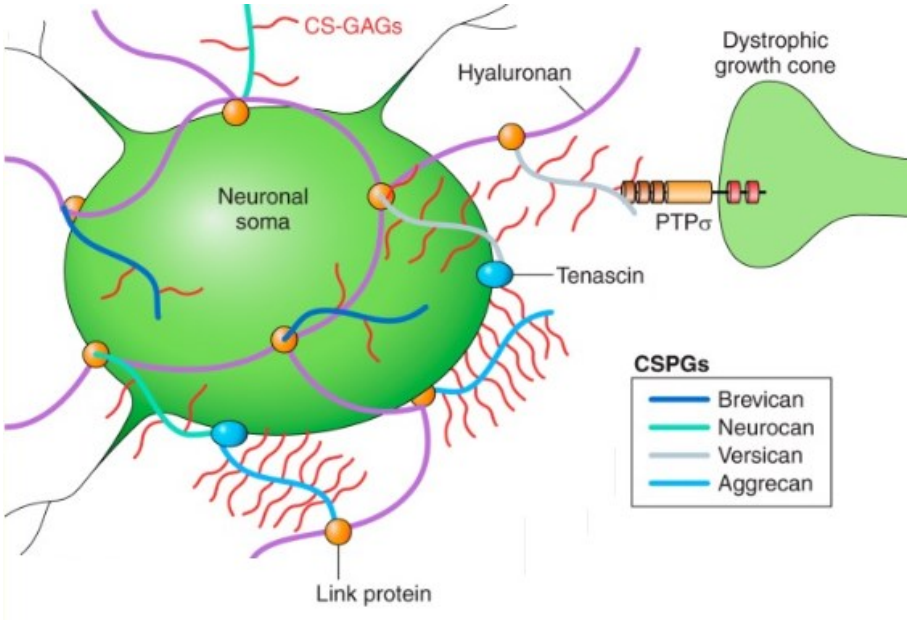

Figure 7 This figure shows binding of PTPσ receptor to the CS (red) of proteoglycans (blue, green and grey) which form the PNN around neuron. When many receptors bind to the CSPGs neuron stops its growth and form dystrophic endbulb. Modified from (Tran, Warren and Silver, 2018)

3.3 Aspects of CSPGs in the treatment of CNS injury

Treatment of traumatic spinal cord and brain injuries is based on enhancement of neuronal plasticity and removal of glial scar components. It is important to note, that formation of glial scar is necessary for successful healing, when it was prohibited, wound still did not heal and there was not scaffold for the neurite growth (Anderson *et al.*, 2016).

One approach to surpass the glial scar is to enzymatically digest prohibitory CS chains on proteoglycans. Enzymes such as chondroitinase ABC and hyalurodinase cleaves them, restore plasticity state and induce neuronal growth (Bradbury *et al.*, 2002). Problem is, that even though neurons can regrow in absence of lecticans and hyaluronan, formation of stable synapses is significantly reduced, since they are normally stabilised by the chondroitin sulphate proteoglycans of PNNs (Corvetti and Rossi, 2005). Also, those enzymes are of bacterial origin, have too broad function for the use in medicine, they can be delivered only directly in site of

treatment and have short half-life. More refined approach to disintegrate PNN at defined place is needed for the injury treatment (Zhao *et al.*, 2011; Tauchi *et al.*, 2012). Lentiviral or AAV (adeno-associated viral) vectors are used as a solution for the prolonged expression of ChABC (Alves *et al.*, 2014; James *et al.*, 2015). Natural mammalian enzymes have advantage, since they cannot start immune system reaction. ADAMTS-4 cleaves all of the lecticans - aggrecan, brevican, versican, neurocan and phosphacan. Exogenous ADAMTS-4 promoted axonal regeneration after SCI and its effect was comparable to the ChABC. Normally present endogenous levels are not enough to facilitate recovery (Tauchi *et al.*, 2012). For the long-term delivery of ADAMTS-4 AAV vector is used, this approach leads to increased neuronal growth and functional recovery (Griffin *et al.*, 2020). Mice with knockout of CS chain forming enzyme, showed greater recovery after SCI, than mice treated with ChABC. CS was not eliminated completely in this knockout mice, its amount was just reduced, because of that there was still some present in order to regulate neuronal growth and new synapse formation (Takeuchi *et al.*, 2013).

Today more attention is focused on proteoglycan modification instead of their cleavage and also on the manipulation of CSPG receptors. Neutralization of proteoglycans in general or specifically 4-sulfated CSPGs with antibodies has the same effect in enhancing neuronal growth as their digestion with ChABC, but it is not site-specific either. Advantage is that those peptides can be designed to be able to pass through blood-brain barrier (Bovolental *et al.*, 1997; Loers *et al.*, 2019). CSPG receptors PTPσ and LAR can be both blocked by exogenous peptides. After their inhibition, recovery of damaged axons is promoted (Dyck *et al.*, 2018). Also, modification of myelin might be helpful. Axons cannot grow through the myelin left at the site of injury. On the other hand, myelin sheath of the growing axon does not prohibit its sprouting and elongation (Li and Strittmatter, 2003). Raisman in his article proposes, that myelin might actually serve as scaffold for axonal growth (Raisman, 2004).

Relaxation of inhibitory effects is not enough to enable complete recovery. Better option is to also deliver growth factors at the site of injury to enhance regrowth. Upregulation of growth factors considerably helps in regeneration, but only at small scale at the site of applied treatment and it does not support long distance growth of axons (Kawaja and Gage, 1991). Nerve growth factor (NGF) enhances sprouting of damaged neurons. They can grow through the scar despite the proteoglycans, but only as far as NGF is present, afterwards they stop (Oudega and Hagg,

1996). Neurotrophic factors enable local functional regeneration of axons as well (Ramer *et al.*, 2002).

Best approach is to combine both suppression of growth inhibitors, such as CS removal and enhancement of growth factors while providing path with the scaffold for the neuron. In study by Tropea *et al.* they dissolved sugar chains in CSPGs by ChABC and enhanced growth of axons by injected neurotrophic factor. Combination of both therapies had better results than either of them alone (Tropea, Caleo and Maffei, 2003).

4 Conclusion

Traumatic brain and spinal cord injuries are occurring worldwide with high frequency and can have long lasting effects on the health or even cause death. Glial scar which forms at the site of injury repairs the blood-brain barrier at the earliest stage of injury, but later prohibits restoration of the functional neuronal network. Understanding of the underlying molecular mechanism of the glial scar extracellular matrix formation and its inhibitory effect is important in order to restore plasticity and induce neuronal growth.

One of the main components of the CNS extracellular matrix are CSPGs, they prohibit plasticity and therefore neuronal growth. But they are also needed in order to stabilize a new synapse. There has to be a balance between growth and stabilization in the healthy organism in order to maintain stable function of the brain, but also to learn and create new memories. CSPGs link together other components and also bind the regulatory molecules. This function is normally present in perineuronal nets and similar ECM structure forms also after injury. CSPGs inhibit neuronal outgrowth mainly thanks to their bound CS chains, which are recognized by inhibitory PTPσ receptor. Aggrecan is CSPG with highest number of bound chondroitin sulphates, its function in healthy organism is to stabilize the ECM in PNNs and prohibit plasticity. In the glial scar, both saccharide chains and protein core of aggrecan prohibit neuronal plasticity. Mechanism behind prohibition of neuronal growth by aggrecan is same both in perineuronal net and glial scar, even though there are differences in the other present ECM components, which might cause different outcome. Neurocan and brevican are inhibiting neuronal plasticity as well, knockout mice strains showed, that their deficiency supports axon recovery.

In order to treat TBI and SCI plasticity state must be restored. There are different approaches trying to either overcome inhibition or restart neuronal growth. Combinatorial strategy gives

the best results and as such they should be more pursued in the future. Currently there are not any procedures, which could be used in human medicine. More research is needed in order to create safe, effective and targeted cure. Several approaches based on CS modification are being researched. One of them is removal of bound saccharides by enzymes. Complication of this treatment, which needs to be overcome is to specifically deliver enzyme at the injury site. Inhibition of PTPσ binding sites by exogenous peptides is another promising option, which should merit more attention in the future. Also changes in sulfation pattern could be an option, more 6-sulfation of CS chains enhances plasticity, in contrast with upregulated 4-sulfation. Future research about artificial sulfation pattern changes in glial scar in order to enhance neuronal growth might be promising. Another direction of research is characterization of lectican protein cores, such as aggrecan. Since even deglycosylated aggrecan inhibits neuronal growth it would be good to find which neuronal receptor is involved in its recognition.

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